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**RESEARCH ARTICLE**

# A COMPARATIVE ANALYSIS OF ANTIBACTERIAL ACTIVITY OF *Citrus limonium* JUICE EXTRACTS, ANTIBIOTICS AND COMMERCIALLY AVAILABLE CITRIC ACID AGAINST NEW STRAINS OF BACTERIA FOR THE PREVENTION OF EYE INFECTIONS

***Khusro A[1], Preetamraj JP[1], Panicker SG[1, 2]**

**1.** Department of Plant Biology and Biotechnology, (PG. Biotechnology), Loyola College, Nungambakkam, Chennai (India).

**2.** Helen Keller Research Centre (HKRC), Chennai (India).

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## *Abstract*

The present study was carried out to find out the antibacterial activity of *Citrus limonium* juice extracts against new strain of pathogenic bacteria isolated from poultry farm. As microorganisms are becoming resistant to antibiotics, our study focuses on the prevention of eye infections caused by new strains of *Bacillus* species by using *Citrus limonium* juice extracts. Different solvent extracts with various concentrations were prepared in order to determine their antibacterial activity against new strains of *Bacillus licheniformis*. Maximum zone of inhibition of 14.5 mm and 12 mm were shown by aqueous extracts of *Citrus limonium* against *Bacillus licheniformis* strain 018 and *B. licheniformis* strain BIHPUR 0104 respectively using Agar disc diffusion method. Aqueous extracts of *Citrus limonium* were effective even at less concentration against *B.licheniformis* strain 018. Ethanol and acetone extracts were less effective against both the strains. 100% *Citrus limonium* extracts were showing more inhibitory action compared to antibiotics (Streptomycin and Nalidixic acid) and commercially available citric acid against *B.licheniformis* strain 018. Antibiotics were more effective compared to *Citrus limonium* extracts against *B. licheniformis* strain BIHPUR 0104 but the citric acid was ineffective against the same strain. Average activity index for antibiotics was found to be more than 0.5 which indicates *Citrus limonium* to be a potential bactericidal agent. From this investigation it is clear that *Citrus limonium* can be used as a potential antibacterial agent against new strains of *B. licheniformis* which causes eye infections in humans.



## Introduction

For a long period of time, plants have been a valuable source of natural products for maintaining human health (Tanaka *et al*., 2006). Herbs, spices and different parts of plants have been used for many years for the prevention and treatment of infections. Plants with antimicrobial properties can be of great significance for the treatment of infections (Nascimento *et al*., 2000). Plant based extracts can be extracted from any part of plant like barks, leaves, fruits, seeds and fruit rinds etc.(Parekh *et al*., 2007). Fruits are one of the oldest forms of food known to humans. Fruit juices are of great demand due to their antibacterial activity and for the treatment of arthritis, heart diseases, muscle aches and drug addiction (Tedesco *et al*., 2001). Hence the fruit juices with the property of bioavailability and retention of certain minerals by polyphenolic compounds can be recommended for their use as an alternative anti-infective agent in natural medicine for the treatment of infectious diseases (Gislene *et al*., 2000). Citrus fruits are acidic fruits which contain flavanones and many polymethoxylated flavones which are very rare in other plants (Ahmad *et al*., 2006). Lemon (*Citrus limonium*) is an important medicinal plant of the family *Rutaceae*. It is

cultivated mainly for alkaloids which are having anticancer and antibacterial activities in different parts viz. leaves, stem, root and flower (Kawaii *et al*., 2000). The active chemical constituents of lemon are limonene, citral, citronellal, linolyl and nerol. Its action also include antisclerotic, antineuralgic, antirheumatic, coagulant, stimulant, insecticide and disinfectant (Grieve *et al*., 2005). Citrus *limonium* is rich in citric acid. Citric acid is both a natural and artificially obtained acid. It is the most frequently used preservative in the world today. It increases the acidity of the bacterial environment making it difficult for it and microbes to survive and, most importantly, to reproduce. A small survey was done on the workers of the poultry farm to know about the infections or diseases they have due to continuous exposure to the bacteria present in the poultry farm. Some of them had digestive problems, stomach pain, body pain and allergy. Very few of them were also found to have sometimes itching in their eyes. *Bacillus licheniformis* is one of the pathogenic bacteria of subtilis group found in poultry farm. *Bacillus licheniformis* is a bacterium that is commonly present in soil and bird's feathers. It is most commonly found around the bird's chest area. This bacterium is one of the major cause of food poisoning and septicaemia. It also causes ophthalmitis (inflammation of eye) in humans whose one of the primary symptom is itching in the eyes. In view of this, the present study was investigated to access the antibacterial activity of different solvent extracts of *Citrus limonium* juice against two new strains of *B. licheniformis* isolated from poultry farm and also to compare the antibacterial activity of crude extracts of *Citrus limonium* juice with two broadly used antibiotics (Streptomycin and Nalidixic acid) and commercially available citric acid for the prevention of eye infection.

## MATERIALS AND METHODS

### Sample collection and isolation

Samples (surface soil) were collected from poultry farm and were brought to the laboratory in aseptic condition. 1 gram of surface soil sample was suspended in 9 ml of saline and mixed vigorously to make uniform suspension. After that soil samples were serially diluted up to $10^{-5}$ and 0.1ml of aliquots were spread over nutrient agar plates from $10^{-4}$ and $10^{-5}$ dilution. The plates were incubated at 37ºC for 24 hours. Pure strains were picked out and purified by repeated streaking on nutrient agar slants. The culture was streaked on slants and kept in incubator at 37°C for 24 hours and were preserved in slants at 4±2°C.

### Biochemical and morphological characterization

Purified isolates were characterized by Biochemical analysis using Indole test, Methyl Red test, Voges Proskauer test, Citrate utilization test, Catalase test and Urease test. Gram staining and Motility test were performed under Morphological test.

### Genomic DNA isolation

2 ml of bacterial culture were centrifuged at 6000 rpm for 5 minutes. The supernatant was discarded. 1 ml of UniFlex™ Buffer 1 and 10 µl of RNase were added to the pellet obtained. Mixed well by pipetting and incubated for 30 minutes at 37ºC in a water bath. To the lysed samples 1 ml of 1:1 phenol:chloroform were added and mixed well. The samples was centrifuged at 10,000 rpm for 15 minutes at room temperature. The aqueous layers were separated in a fresh 1.5 ml vial. To the aqueous layer 1 ml of UniFlex™ Buffer 2 were added and mixed well by pipetting. The mixture was centrifuged at 12,000 rpm for 15 minutes at room temperature. The supernatant was discarded. To the pellet 500 µl of 70% ethanol were mixed. Again it was centrifuged at 10,000 rpm for 10 minutes at 4ºC. The supernatant was discarded. The pellet was air dried for about 10-15 minutes till the ethanol evaporate. The pellet was resuspended in 50-100 µl of UniFlex™ Elution Buffer. DNA was stored at -20ºC.

### PCR amplification and sequencing

The 16S ribosomal RNA was amplified by using the PCR (ependorfep.Gradient) with *Taq* DNA polymerase and primers 27F (5` AGTTTGATCCTGGCTCAG 3`) and 1492R (5`ACGGCTACC TTGTTACGACTT 3`). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, primer annealing at 52°C for 1 min and primer extension at 72°C for 1 min. At the end of the cycling, the reaction mixture was held at 72°C for 10 min and then cooled to 4°C. PCR amplification was detected by agarose gel electrophoresis and visualized by alpha image gel doc after ethidium bromide staining. The PCR product obtained was sequenced by an automated sequencer (Genetic Analyzer 3130, Applied Biosystems, and

USA). The same primers as above were used for sequencing. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST available at http:// www.ncbi-nlm-nih.gov/. 16S rRNA sequence was then submitted to the GenBank, NCBI, USA.

**Preparation of aqueous, ethanolic and acetone extracts of *Citrus limonium***

The fresh citrus fruit (*Citrus limonium*) was purchased from local market of Nungambakkam, Chennai (India). The procured fruit was washed and dried at room temperature. The outer portion of *Citrus limonium* was wiped with 70% alcohol and fresh juice was collected by juicer machine. After that it was sieved through 8 layers of sterile mesh cloth. These filtered extracts were centrifuged at 6000 rpm for 10 minutes. Supernatants were collected as 100% extracts having pH of 3.5. These extracts were stored at 4°C in refrigerator for further use. Extracts of *Citrus limonium* were diluted to make different concentrations such as 75%, 50%, 25% and 5% by mixing with appropriate volume of sterile double distilled water. The ethanolic and acetone extracts were prepared following the same procedure with the exception of solvent which was 95% ethanol and acetone instead of sterilized double distilled water.

**Preparation of Citric acid**

The commercially available citric acid was prepared in the concentration of 30 µg/disc by appropriate mixing of sterilized double distilled water. pH was maintained equivalent to that of crude *Citrus limonium* juice (pH = 3.5).

**Microorganisms used**

*Bacillus licheniformis* strain 018 (Accession no.-KC342225) and *Bacillus licheniformis* strain BIHPUR 0104 (Accession no.- KC424492) isolated from poultry farm were used.

**Antibacterial sensitivity test using disc diffusion method**

The microorganisms (*Bacillus licheniformis* strain 018 and *Bacillus licheniformis* strain BIHPUR 0104) grown in Nutrient broth were transferred to Mueller Hinton Agar plates with the help of sterile cotton swabs. 25 µl of pure extracts, aqueous, ethanolic and acetone extracts of *Citrus limonium* were aseptically transferred to each discs (6mm) at all dilutions that were made in triplicate. 25 µl of sterilized double distilled water, 95% ethanol and acetone were added in sterile discs as negative control in aqueous, ethanolic and acetone extract plates respectively. The soaked discs were transferred aseptically to the plates seeded with the microorganisms with the help of ethanol dipped and flamed forceps. The petriplates were incubated in upright position at 37°C for 24 hours. After 24 hours zone of inhibition formed by different solvent extracts of garlic at different concentrations against the tested microbes were measured. The mean and standard deviation of the diameter of zone of inhibition were calculated.

**Antibiotic sensitivity testing**

The test microorganisms were also tested for their sensitivity against the antibiotic Streptomycin (10 µg) and Nalidixic acid (30 µg). Antimicrobial activities of 100% *Citrus limonium* extracts and commercially available citric acid were performed to compare with the zone of inhibition obtained by antibiotics. A homogeneous bacterial lawns were prepared on Mueller Hinton agar plates using sterile cotton swabs. The sterile discs of 6 mm diameter were soaked with 25 µl of 100% *Citrus limonium* extracts, commercially available citric acid and Nalidixic acid. Using an ethanol dipped and flamed forceps the standard antibiotic discs of Streptomycin (S), soaked discs of *Citrus limonium* extracts, Nalidixic acid (NA) and Citric acid were aseptically placed over the agar plates sufficiently separated to avoid overlapping of zone of inhibition. Plates were incubated at 37°C for 24 hours. After 24 hours, diameters of zone of inhibition were measured in mm and results were recorded.

**Determination of Activity index**

Following formula was used to determine Activity index

$$\text{Activity index} = \frac{\text{Zone of inhibition of extract}}{\text{Zone of inhibition of antibiotic}}$$

## Results

In this study the microorganisms isolated from poultry farm were identified as new strains of *Bacillus* species according to Morphological, Biochemical characteristics and 16S rRNA gene sequencing. Antibacterial activities of different solvent extracts of *Citrus limonium* at different concentrations were determined by Agar disc diffusion

method against new strains of *Bacillus* species isolated from poultry farm. *Citrus limonium* was found to be active against both the strains i.e. *Bacillus licheniformis* strain 018 and *Bacillus licheniformis* strain BIHPUR 0104. The aqueous extracts of *Citrus limonium* were found to be more effective compared to ethanolic and acetone extracts against both the new strains (Table 1 and 2). *B.licheniformis* strain 018 were more susceptible compared to *B.licheniformis* strain BIHPUR 0104 against *Citrus limonium* extracts. Maximum zone of inhibition shown by aqueous extracts of *Citrus limonium* was 14.5 mm and 12 mm against *B.licheniformis* strain 018 (Fig.-1) and *B.licheniformis* strain BIHPUR 0104 (Fig.-2) respectively. The aqueous extracts of *Citrus limonium* were found to be effective against *B.licheniformis* strain 018 even at 25% concentration with 9 mm of zone of inhibition. The same aqueous extracts were ineffective against *B.licheniformis* strain BIHPUR 0104 at 25% concentration. Ethanolic extracts of *Citrus limonium* were found to be ineffective at 25% concentration against *B.licheniformis* strain 018. Minimum and maximum zone of inhibition shown by ethanolic extracts were 8.5 mm and 13 mm at 50% and 100% of concentration respectively (Fig.-3). *B.licheniformis* strain BIHPUR 0104 were also found to be resistant to ethanolic extracts of *Citrus limonium* at 25% concentration and were showing minimum and maximum zone of inhibition 9.3 mm and 11 mm at 50% and 100% concentration respectively (Fig.-4). Minimum zone of inhibition of 8 mm and 2 mm was shown by acetone extracts of *Citrus limonium* among all the extracts tested against *B.licheniformis* strain 018 and *B.licheniformis* strain BIHPUR 0104 respectively at 50% concentration (Fig.-5 and 6). *B.licheniformis* strain BIHPUR 0104 were found to be more susceptible to acetone extracts compared to ethanolic extracts at 50% and 100% concentration whereas the ethanolic extracts of *Citrus limonium* were showing more zone of inhibition compared to acetone extracts at all the concentrations against *B. licheniformis* strain 018. The diameter of zone of inhibition obtained by *Citrus limonium* at 100% concentration by disc diffusion method was also compared to those obtained against two standard antibiotics and commercially available citric acid (Table-3). *Citrus limonium* extracts were showing wider zone of inhibition of 14.5 mm compared to Streptomycin for *B.licheniformis* strain 018. Nalidixic acid and commercially available citric acid were found to be ineffective against the same strain (Fig.-7). *B.licheniformis* strain BIHPUR 0104 were susceptible to *Citrus limonium* extracts, Streptomycin and Nalidixic acid with wider zone of inhibition except commercially available citric acid. Nalidixic acid was found to be more effective compared to Streptomycin and 100% *Citrus limonium* extracts (Fig.-8). *B.licheniformis* strain 018 were more susceptible to 100% *Citrus limonium* extracts than antibiotics and commercially available Citric acid. *Citrus limonium* extract was less effective compared to antibiotics against *B.licheniformis* strain BIHPUR 0104. The zone of inhibition shown by Streptomycin and Nalidixic acid was measured and average activity index of *Citrus limonium* for *B.licheniformis* strain 018 and *B.licheniformis* strain BIHPUR 0104 was calculated (Table-4). The average activity index for Streptomycin and Nalidixic acid against *B.licheniformis* strain 018 were found to be 1.11 and 14.5 respectively. For *B.licheniformis* strain BIHPUR 0104, *Citrus limonium* had the average activity of 0.66 and 0.64 against Streptomycin and Nalidixic acid respectively.

**Table 1: Antibacterial activity of different solvent extracts of *Citrus limonium* at different concentrations against *Bacillus licheniformis* strain 018 by disc diffusion method (in mm).**

| Concentration | Aqueous extracts | Ethanol extracts | Acetone extracts |
|---|---|---|---|
| 5% | - | - | - |
| 25% | 09±0 | - | - |
| 50% | 11±1.41 | 8.5±0.7 | 08±0 |
| 75% | 12.5±0.7 | 10.5±0.7 | 10±0 |
| 100% | 14.5±0.7 | 13±1.41 | 11.6±0.57 |

**Table 2: Antibacterial activity of different solvent extracts of *Citrus limonium* at different concentrations against *Bacillus licheniformis* strain BIHPUR 0104 by disc diffusion method (in mm).**

| Concentration | Aqueous extracts | Ethanol extracts | Acetone extracts |
|---|---|---|---|
| 5% | - | - | - |
| 25% | - | - | - |
| 50% | 9.5±0.7 | 9.3±0.57 | 02±0 |
| 75% | 11.5±0.7 | 10±0 | 10.6±0.57 |
| 100% | 12±0 | 11±0 | 11.3±0.57 |

**Table 3: Comparison of the antibacterial activity of *Citrus limonium* extracts, standard antibiotics and commercially available citric acid (in mm).**

| Bacterial strains | Streptomycin (S) | Nalidixic acid (NA) | Citric acid | *Citrus limonium* (100%) |
|---|---|---|---|---|
| *B. licheniformis* strain 018 | 13±0 | - | - | 14.5±0 |
| *B. licheniformis* strain BIHPUR 0104 | 18±1.41 | 18.5±0.7 | - | 12±0.7 |

**Table 4: Average Activity index of *Citrus limonium* for *B. licheniformis* strains**

| Bacteria | Zone of inhibition of 100% *C.limonium* (mm) | Activity index for Streptomycin (S) | Activity index for Nalidixic acid (NA) |
|---|---|---|---|
| *B. licheniformis* strain 018 | 14.5±0 | 1.11 | 14.5 |
| *B. licheniformis* strain BIHPUR 0104 | 12±0.7 | 0.66 | 0.64 |

Fig: 1- Aqueous extract (Strain 018)

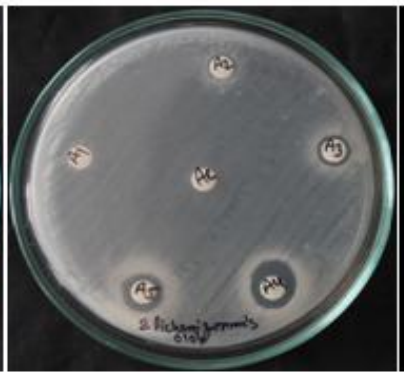

Fig:2- Aqueous extract (Strain BIHPUR 0104)

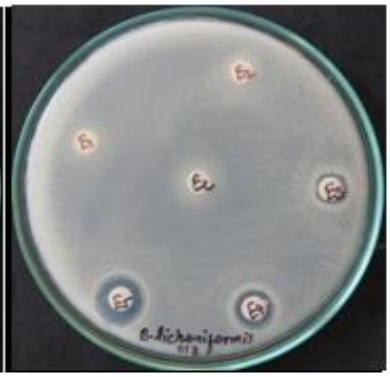

Fig:3- Ethanol extract (Strain 018)

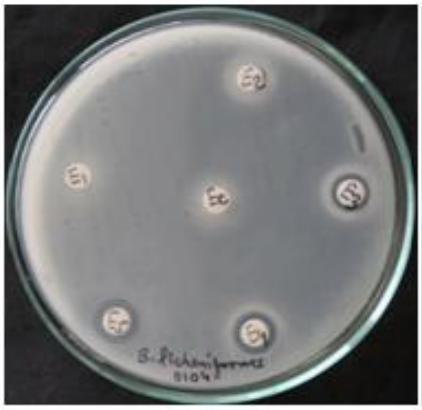

Fig:4- Ethanol extract (Strain BIHPUR 0104)

Fig:5- (Acetone extract) (Strain 018)

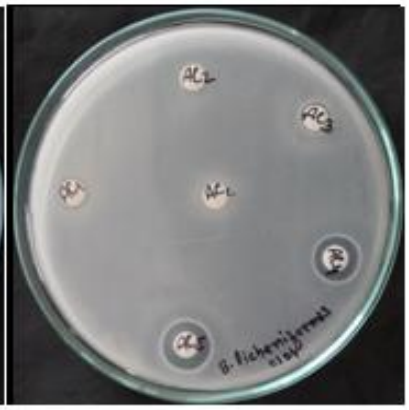

Fig:6- (Acetone extract) (Strain BIHPUR 0104)

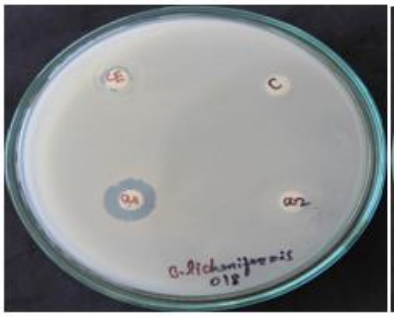

Fig:7- (Antibiotic test)
(Strain 018)

Fig:8- (Antibiotic test)
(Strain BIHPUR 0104)

($A_1$, $E_1$, $AC_1$= 5% Concentration; $A_2$, $E_2$ and $AC_2$= 25% Concentration; $A_3$, $E_3$ and $AC_3$= 50% Concentration; $A_4$, $E_4$ and $AC_4$= 75% Concentration; $A_5$, $E_5$, $AC_5$ and CE= 100% Concentration; Ac, Ec and ACc= Negative control; C= Citric acid; $a_1$= Streptomycin; $a_2$= Nalidixic acid)

## DISCUSSION

Conte *et al* (2007) reported that lemon extracts were showing inhibitory action even at low concentration against *Bacillus licheniformis* compared to other *Bacillus* species. In this investigation different solvent extracts of *Citrus limonium* juice were also found to be effective against two new strains of *Bacillus licheniformis* isolated from poultry farm. Aqueous extracts of *Citrus limonium* were showing zone even at 25% concentration against *B. licheniformis* strain 018. Kumar *et al* (2012) determined that juice of lemon were less effective against *Bacillus* species. In the present study *Citrus limonium* juice extracts were showing moderate zone of inhibition. Saeed *et al* (2006) also reported the effect of *Citrus limonium* on 25 different strains of *Bacillus* species with maximum zone of inhibition. According to Kumar *et al* (2011), ethyl acetate and ethanol peel extracts of *Citrus limonium* were more effective compared to aqueous extracts against *Bacillus* species. The peel extracts of *Citrus limonium* were also found to be effective against Metacillin and Penicillin. That study is not accordance with our finding. In our investigation aqueous extracts of *Citrus limonium* juice were more effective compared to ethanol and acetone extracts against new strains of *Bacillus* species isolated from poultry farm. The aqueous extracts showed better result compared to ethanol and acetone extracts. This may be because very less active compounds of *Citrus limonium* juice were dissolved in ethanol and acetone. The antimicrobial activity of 100% *Citrus limonium* extracts was found more effective compared to Streptomycin and Nalidixic acid against *B. licheniformis* strain 018. On the other hand the antibiotics were showing more or less the same inhibitory effect compared to 100% *Citrus limonium* juice extracts against *B. licheniformis* strain BIHPUR 0104. The aqueous, ethanol and acetone extracts were showing significant increase in zone of inhibition as the concentration increased. This finding agrees with the work of Safithri *et al* (2011) who showed that the higher concentration of organic solvent extracts of medicinal plant, the more bacteria were inhibited to grow, as indicated by the larger zone of inhibition. In the present investigation ethanol and acetone extracts of *Citrus limonium* showed more or less the same antibacterial activity. Aqueous extracts showed maximum antibacterial activity when compared to other solvents against both the new strains of *Bacillus licheniformis*. This shows that water has the capability to extract one such antibacterial agent which may be very toxic to these two new strains. So it indicates that different extracts may have diverse antibacterial agent that has different mode of action. The differences in the zone of inhibition for their antibacterial activity with the same source when extracted with different solvents has proven that all phytochemical that are responsible for antibacterial activity are not soluble in a single solvent. Hence solvents of different polarity should be employed as discussed in this study (Polar- water, acetone and ethanol). Upadhyay *et al* (2010) and Prabuseenivasn *et al* (2006) reported the effectiveness of citrus fruits essential oil against *Bacillus* species. In this investigation the inhibitory effect of *Citrus limonium* juice were observed against new strains of *Bacillus licheniformis*. *Citrus limonium* juice extracts showed better effectiveness than antibiotics against Gram(+) bacteria due to absence of lipo-polysaccharide layer in Gram(+) bacteria that might function as an effective barrier against any incoming biomolecules (Inouye *et al*.,2001). There might be another possibility that citrus fruits increase the plasma membrane permeability which results into death of bacterial cells after massive ion leakage (Lambert *et al*., 2001 and Walsh *et al.*, 2003). Our study favors this finding. 100% *Citrus limonium* extracts were more effective than the antibiotics (Streptomycin and Nalidixic acid) against *B.*

*licheniformis* strain 018. Nalidixic acid were not showing zone of inhibition against this strain. *B. licheniformis* strain BIHPUR 0104 were found to be more resistant to 100% *Citrus limonium* extracts compared to the antibiotics. This may be due to the reason that there is less use of antibiotics in poultry farm which resulted one of these two strains to be less resistance to these antibiotics. In this investigation the commercially available citric acid were found to be ineffective against these two new strains of *Bacillus licheniformis*. It clearly indicates that citric acid is not the only component in the *Citrus limonium* juice for causing its bactericidal activity. There may be some other active compounds present in the *Citrus limonium* juice which inhibits the growth of these two new strains of bacteria. *Citrus limonium* juice was used without any purification and then also they were showing good activity index (more than 0.5). So the result suggests the *Citrus limonium* juice extracts to be a potential antibacterial agent. The emergence of drug resistance microbes needs for an antimicrobial agent with higher or similar antibiotic beneficial properties. As *B. licheniformis* strain 018 and *B. licheniformis* strain BIHPUR 0104 are pathogenic to humans causing ophthalmitis so *Citrus limonium* can be a good substitute of antibiotics for the prevention and treatment of eye infections to the people who are continuously exposed to these two bacteria in the poultry farm. As citric acid present in *Citrus limonium* is an irritant and may cause injuries to the eyes so sprayer can be made to spray in the environment and surface of the poultry farm to inhibit the growth of these two new strains of *Bacillus licheniformis* instead of creating eye drops so that workers of the poultry farm can be prevented from the eye infections caused by these strains. Drugs derived from plants are effective and rarely have side effects. If the active components of *Citrus limonium* (excluding citric acid) having antibacterial activity can be isolated then the new drugs having no side effects to the eyes can be synthesized for the treatment of eyes infections. Apart from this, disinfectants solutions and hand lotions can be made from the active components of *Citrus limonium* juice which will prevent the people from the eyes infections caused by new strains of *B. licheniformis* after touching the birds of the poultry farm. Results of present investigation suggest antibacterial activity of *Citrus limonium* juice extract against two new strains of *B. licheniformis* and indicates the inhibitory action of *Citrus limonium* juice extracts compared to standard antibiotics and commercially available citric acid. So *Citrus limonium* offers a new source of antibacterial agent for the prevention of eye infections. From this result it is clear that medicinal value of *Citrus limonium* juice extracts is comparable to antibiotics and commercially available citric acid.

## CONCLUSION

The present investigation emphasizes antibacterial properties of *Citrus limonium* juice extracts against new strains of pathogenic bacteria causing eye infections in humans. The data in this study support the use of *Citrus limonium* for the treatment of diseases, especially the prevention of eye infections where access to antibiotics is restricted. The fruit has an extra-ordinary potential to yield active compounds which could be valuable for the prevention and treatment of eye infections and other diseases. *Citrus limonium* may be a good source to decrease the burden of drug resistance bacteria and to reduce the use of antibiotics in the poultry farm. More importantly, scientific study of *Citrus limonium* fruits is needed to determine their pharmaco-kinetics properties and to provide evidence for their efficacy.

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